

# Insect growth regulators as potential insecticides to control olive fruit fly (*Bactrocera oleae* Rossi): insect toxicity bioassays and molecular docking approach

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## Abstract

**BACKGROUND:** Olive fruit fly, *Bactrocera oleae* (Rossi), is a key pest in olive orchards, causing serious economic damage. To date, the pest has already developed resistance to the insecticides commonly applied to control it. Thus, in searching for new products for an accurate resistance management programme, targeting the ecdysone receptor (EcR) might provide alternative compounds for use in such programmes.

**RESULTS:** Residual contact and oral exposure in the laboratory of *B. oleae* adults to the dibenzoylhydrazine-based compounds methoxyfenozide, tebufenozide and RH-5849 showed different results. Methoxyfenozide and tebufenozide did not provoke any negative effects on the adults, but RH-5849 killed 98-100% of the treated insects 15 days after treatment. The ligand-binding domain (LBD) of the EcR of *B. oleae* (BoEcR-LBD) was sequenced, and a homology protein model was constructed. Owing to a restricted extent of the ligand-binding cavity of the BoEcR-LBD, docking experiments with the three tested insecticides showed a severe steric clash in the case of methoxyfenozide and tebufenozide, while this was not the case with RH-5849.

**CONCLUSION:** IGR molecules similar to the RH-5849 molecule, and different from methoxyfenozide and tebufenozide, might have potential in controlling this pest.

**Keywords:** *Bactrocera oleae*; tephritid pest; ecdysone agonists; modelling; docking; potential control

## 1 INTRODUCTION

The olive fruit fly, *Bactrocera* (*Dacus*) *oleae* (Rossi) (Diptera: Tephritidae), is considered to be the key pest of the Mediterranean Basin olive orchards.<sup>1</sup> Females lay their eggs in both green and ripening olive fruit, and larvae feed upon the pulp of the fruit. They finally pupate inside the olive or exit to pupate on the ground.<sup>2</sup> This pest causes a reduction in yield owing to a premature fruit drop or a loss of weight of the fruit caused by feeding larvae. Furthermore, microorganism growth inside the fruit increases the acidity of olive oils, which decreases their quality and commercial value. In table olives, *B. oleae*'s damage totally reduces their commercial value.<sup>2,3</sup> Control methods against this pest include bait sprays, cover sprays and mass trapping.<sup>1</sup> Traditional insecticides, such as organophosphates, and other more recently developed compounds, such as spinosad, are commonly applied as bait sprays. However, *B. oleae* has the potential to develop resistance to some of these compounds, as has already been demonstrated in the case of dimethoate and spinosad.<sup>2,4</sup> Therefore, evaluating alternative treatments against this pest is necessary if an effective resistance management programme is to be developed. Insect growth regulators (IGRs) such as the ecdysone agonists, which are selective and have already been applied against other tephritid pests, could be an alternative.<sup>5</sup> These IGRs work by binding specifically on

the ecdysone receptor (EcR) of susceptible insects (Lepidoptera, Coleoptera and Diptera, especially in mosquitoes).<sup>6–8</sup> Typically, they induce premature lethal moulting in larval stages of different insect orders, and they affect reproduction, reducing egg production, producing ovicidal activity and disrupting normal spermatogenesis processes.<sup>9–11</sup> The best examples of this group of IGRs are the dibenzoylhydrazine (DBH)-based tebufenozide [RH-5992; 3,5-dimethylbenzoic acid 1-(1,1-dimethylethyl)-2-(4-ethylbenzoyl)hydrazine; *N*-*tert*-butyl-*N'*-(4-ethylbenzoyl)-3,5-dimethylbenzohydrazide], methoxyfenozide [RH-2485; 3-

methoxy-2-methylbenzoic acid 2-(3,5-dimethylbenzoyl)-2-(1,1-dimethylethyl)hydrazine; *N*-*tert*-butyl-*N'*-(3-methoxy-*o*-toluoyl)-3,5-xylohydrazide] and the unsubstituted RH-5849 (1-*tert*-butyl-1,2-dibenzoyl hydrazine), which contains no substitutions on the two benzoyl rings (A and B).

The EcR is the receptor of the insect moulting hormone 20-hydroxyecdysone (20E). Binding between receptor and ligand starts the hormone signalling cascade regulating important physiological events in an insect's life, such as growth, metamorphosis and reproduction. EcR works together with a heterodimer partner ultraspiracle (USP), also named RXR. Both proteins belong to the superfamily of nuclear receptors. Typically, these receptors consist of a modular structure with a highly conserved DNA-binding domain (DBD) and a ligand-binding domain (LBD). The latter is composed of 12  $\alpha$ -helices (H1 to H12) arranged as an antiparallel three-layer sandwich associated with a hairpin of two short  $\beta$ -strands ( $\beta$ 1 and  $\beta$ 2) in the loop between helices H5 and H6.<sup>12</sup> The ligand-binding pocket that holds the cognate ligand is formed by side chains of residues from helices H3, H5, H7, H11 and H12, and the  $\beta$ -hairpin.<sup>11–17</sup>

In the present study, the insecticidal activities of three ecdysone agonists, methoxyfenozide, tebufenozide and RH-5849, on *B. oleae* adults were tested. Data were also compared with currently used insecticides such as dimethoate and spinosad. Adults were exposed to an inert treated surface or were forced to ingest insecticide via their drinking water. Following this, the LBD of the EcR of this pest (BoEcR-LBD) was cloned and sequenced. Subsequently, a three-dimensional model of the BoEcR-LBD was constructed to evaluate whether it exhibited the typical canonical structure. With this, an investigation was made of the extent to which the DBH-based ecdysone agonist molecules can fit in the ligand-binding pocket. The latter molecular docking data can help to explain the range of insecticidal activities observed in the bioassays conducted with *B. oleae* adults.

## 2 MATERIALS AND METHODS

### 2.1 Insects

*Bactrocera oleae* adults were obtained from infested olive fruit collected from different olive orchards in Madrid, Cáceres and Jaén (Spain). Fruit was returned to the Laboratory of Entomology of the Technical University of Madrid (Spain) and placed on plastic grilles placed on the top of methacrylate cages. When larvae were close to pupariation, they egressed from the fruit into the cages and the puparia were collected. Adults emerging from these pupae were used in the study.

### 2.2 Insect bioassays

Bioassays were carried out in a controlled environment chamber ( $25 \pm 2^\circ\text{C}$ ,  $75 \pm 10\%$  RH and 16:8 L:D photoperiod). The following active ingredients were tested: methoxyfenozide (Runner<sup>®</sup>, 24 SC; Dow Agrosciences, Madrid, Spain), tebufenozide (Mimic 2F<sup>®</sup>, 24 SC; Dow Agrosciences, Madrid, Spain), RH-5849 (technical product, >95%; Rohm and Haas, Spring House, PA), dimethoate (Dimetoato 40 Progress<sup>®</sup>, 40 EC; Cheminova Agro SA, Madrid, Spain) and spinosad (Spintor-Cebo<sup>®</sup>, 0.024 CB; Dow Agrosciences, Madrid, Spain). Distilled water was used as a control. Solutions of the products were prepared freshly in distilled water prior to the assays, based on their respective maximum field recommended concentrations (MRFC) in accordance with the Spanish registration. Distilled water was used as a control. For RH-5849, 5 mL of acetone per 100 mL solution was used as solvent.

Experiments consisted of five replicates per treatment, each comprising ten adults with an age of <48 h. Distilled water was provided *ad libitum* in small glass vials (15 mm diameter, 22 mm height) covered with Parafilm<sup>®</sup> with a piece of Spontex<sup>®</sup> wiper leading out of the solution to provide a wick for the insects to drink from. Diet (consisting of icing sugar: hydrolysed protein; 4:1) was supplied in small plastic stoppers (24 mm diameter, 6 mm height). Mortality was recorded daily as the percentage of dead flies. Lifespan was measured as the average number of days for which the insects survived per replicate. Reproductive parameters were not evaluated because it was not possible to offer non-damaged olive fruit or an artificial substrate to females for ovipositing.

**Exposure via ingestion.** The oral toxicity of the insecticides was evaluated by offering the solutions via drinking water. Adults were placed into plastic cages (12 cm diameter by 5 cm height, with a 5.5 cm diameter ventilation hole covered with mesh on the top of the cage for ventilation). Insecticide solutions were offered in the same glass vials as those described above. When adults had consumed the insecticide solutions prepared on the first day of the experiment, distilled water was provided.

**Residual contact on glass surfaces.** To evaluate the residual contact activity of the insecticides, glass plates (12 × 12 × 0.5 cm) were treated using a Potter precision spray tower (Burkard Manufacturing Co., Rickmansworth, Herts, UK). Test units were built as soon as the glass plates were dry. They consisted of a round methacrylate frame (10 cm diameter, 3 cm height) and the two square glass plates described above. The plastic frame had eight holes (1 cm diameter): seven covered by a mesh for ventilation, and the last one holding a small rubber tube with a hypodermic needle at the end. The needle was connected to a larger rubber tube providing a continuous flow of air produced by an aquarium pump to assure forced ventilation. Adults were introduced into each test unit, which were then mounted with two rubber bands crossed to hold the structure together. Fungal contamination inside the cages 4 weeks after the treatment made it impossible to evaluate lifespan in this case.

Data (mean values  $\pm$  SE) were subjected to one-way analysis of variance (ANOVA), and Fisher's least significant difference (LSD) test was used to compare responses at field rate concentrations. All statistical analyses were performed using Statgraphics<sup>®</sup> v.5.1.<sup>18</sup> When needed, data were transformed to  $\arcsin \sqrt{(x/100)}$  or  $\log(x + 1)$ , depending on whether the data were expressed as percentages or not, even though non-transformed data are shown in Table 1. If any of the assumptions of the analysis of variance were violated after appropriate transformations, the non-parametric Kruskal–Wallis test was applied. Median values were considered to be significantly different if 95% confidence intervals of medians did not overlap.

### 2.3 Sequencing of BoEcR-LBD and phylogenetic analysis

Total RNA was extracted from freshly collected *B. oleae* olive flies using TRI reagent (Sigma-Aldrich, Bornem, Belgium), based on a single-step liquid-phase separation method.<sup>19</sup> The quality and quantity of the extracted RNA were examined by gel electrophoresis and spectrophotometry using a Nanodrop<sup>™</sup> ND-1000 (Thermo Fisher Scientific, Asse, Belgium). Subsequently, first-strand cDNA synthesis was performed using SuperScript<sup>™</sup> II reverse transcriptase (Invitrogen, Merelbeke, Belgium) with the oligo(dT)<sup>12–18</sup> primers according to the manufacturer's protocol.

The LBD of the EcR from *B. oleae* (BoEcR-LBD) coding sequence was then determined through a number of PCR reaction steps. Partial sequences of the LBD were obtained using degenerate and

**Table 1.** Toxicity of methoxyfenozide, tebufenozide and RH-5849 against olive fly *Bactrocera oleae* adults<sup>a</sup>

Compounds	Concentration (mg AI L <sup>-1</sup> )	% Mortality, 24 h <sup>b</sup>	% Mortality, 7 days <sup>b</sup>	% Mortality, 15 days <sup>b</sup>	Lifespan <sup>c</sup>
<i>Ingestion</i>					
Control	–	0.0 ± 0.0 a	2.5 ± 2.5 a	2.5 ± 2.5 a	79.7 ± 12.2 a
Methoxyfenozide	96	2.5 ± 2.5 a	2.5 ± 2.5 a	2.5 ± 2.5 a	86.0 ± 4.1 a
Tebufenozide	180	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	66.9 ± 7.3 a
RH-5849	180	0.0 ± 0.0 a	31.8 ± 8.1 b	98.2 ± 1.8 b	8.7 ± 0.8 b
		$K = 3.0$ $P = 0.391621$	$F_{3,16} = 15.29$ $P = 0.001$	$F_{3,16} = 589.7$ $P < 0.0001$	$F_{3,16} = 4.16$ $P < 0.0001$
<i>Residual contact</i>					
Control	–	2.0 ± 2.0 a	4.0 ± 4.0 ab	45.8 ± 16.3 a	–
Methoxyfenozide	96	0.0 ± 0.0 a	0.0 ± 0.0 a	49.1 ± 14.4 a	–
Tebufenozide	180	0.0 ± 0.0 a	18.0 ± 5.8 b	58.0 ± 11.6 a	–
RH-5849	180	0.0 ± 0.0 a	86.0 ± 7.5 c	100.0 ± 0.0 b	–
		$K = 3.0$ $P = 0.391621$	$F_{3,16} = 60.63$ $P < 0.0001$	$F_{3,16} = 4.16$ $P = 0.0234$	–

<sup>a</sup> Data are expressed as mean data ± standard error. When followed by the same letter, these are not significantly different (ANOVA, LSD;  $P \geq 0.05$ ).

<sup>b</sup> The systemic insecticide dimethoate, as commercial standard, caused 100% mortality at 24 h after the treatment.

<sup>c</sup> Lifespan was measured as the average number of days for which the insects survived per replicate.

specific primers located in the coding sequence of the LBD and the DBD of the receptor and designed using Primer3 software.<sup>20</sup> Degenerate primers, 5′-TTBYTNGMSGARATMTGGGACGT-3′ and 5′-GAAGTVATGATGYTNMGNATG-3′, were designed on the basis of known EcR sequences from different Mecoptera, Trichoptera, Strepsiptera, Coleoptera, Hymenoptera, Lepidoptera and Diptera insect species. Gene-specific primers were designed in the partial sequence obtained with the degenerate primers. The pairs of primers 5′-GCAGTGAGGAGAGCGTATT-3′ and 5′-GVCGVAARTGYCARGAGTG-3′ and the 5′-CGCAGTTCAGTTAGTATGGA-3′ and 5′-CCACAAGAGGATCAAATCAC-3′ were used to sequence the rest of the BoEcR-LBD. PCR products were purified using the Cycle Pure kit (Omega Bio-Tek, Beaver Ridge Circle, Norcross, GA) and were sent for sequencing (AGOWA, Berlin, Germany). Afterwards, the whole fragment was cloned and sequenced for confirmation.

The same *B. oleae* cDNA as used in the identification of EcR-LBD was used for the initial PCR reactions of the cloning. After purification, the PCR products were ligated into a pGEM-T vector (Promega, Madison, WI) according to the manufacturer's instructions. Afterwards, plasmids were transformed in competent *Escherichia coli* XL-1 Blue Cells by heat shock and then plated out on an ampicillin-containing LB (lysogeny broth) agar plate. After 16 h incubation, formed colonies were checked by colony PCR, and several of these positive colonies were then purified using a Plasmid Mini Prep kit (Omega Bio-Tek) and sent for sequencing (AGOWA).

The EcR-LBD sequences of several arthropods and two human orthologues of EcR were retrieved by Blast searches against the GenBank database. The chosen sequences were then aligned by CLUSTALW2/CLUSTALX2, and the phylogenetic trees were made using MEGA4 software.<sup>21,22</sup> Bootstrap analysis with 1000 replicates for each branch position was used to assess support for nodes in the tree.<sup>23</sup>

## 2.4 Modelling of BoEcR-LBD and docking studies

Homology modelling of BoEcR-LBD was performed with the YASARA Structure program running on a 2.53 GHz Intel core duo

Macintosh computer.<sup>24</sup> Different models were built from the X-ray crystal structure coordinates of the EcR-LBD of the lepidopteran *Heliothis virescens* F. in complex with synthetic DAH-based ligand as BYI-06830 (PDB code 3IXP), the EcR-USP receptor of the coleopteran *Tribolium castaneum* Herbst. bound to ponasterone A (PoA) (PDB Code 2NXX),<sup>25</sup> the EcR-LBD of the hemipteran *Bemisia tabaci* Gennadius complexed to PoA (PDB code 1Z5X),<sup>12</sup> the EcR-USP of *H. virescens* in complex with 20E (PDB code 2R40)<sup>26</sup> and the human RXR $\alpha$  (PDB code 3FC6). Finally, a hybrid model was built up from the five previous models.<sup>27</sup> PROCHECK was used to assess the geometric quality of the three-dimensional model.<sup>28</sup> In this respect, the residues of BoEcR-LBD were correctly assigned on the allowed regions of the Ramachandran plot, except for residue Ala75, which occurs in the non-allowed region of the plot (result not shown). Using ANOLEA to evaluate the models,<sup>29</sup> a single residue of BoEcR-LBD over 235 exhibited an energy over the threshold value. This residue is located in a loop region connecting  $\alpha$ -helices. Molecular diagrams were drawn with YASARA and PyMol (DeLano WL, <http://pymol.sourceforge.net>). Docking of 20E, PoA, tebufenozide, methoxyfenozide and RH-5849 to BoEcR-LBD was performed with the YASARA structure program. Clipping planes of BoEcR-LBD complexed to 20E, PoA, tebufenozide, methoxyfenozide and RH-5849 were rendered with PyMol.

## 3 RESULTS

### 3.1 Insect bioassays

As shown in Table 1, neither residual contact nor ingestion exposure of adults to methoxyfenozide, tebufenozide or RH-5849 caused any deleterious effect on adults 24 h after exposure ( $P > 0.05$ ). In contrast, dimethoate and spinosad killed 100 and 62% of adults, respectively, within the first 24 h of treatment (data not shown). Then, in the continuation of the experiment, tebufenozide and methoxyfenozide were not toxic to *B. oleae*. In marked contrast, RH-5849 provoked a significantly higher mortality, and 86% of adults were killed after 7 days of contact with the product, and 100% on day 15. When the RH-5849 was





**Figure 1.** Sequence alignment of ecdysone receptor ligand-binding domains (LBDs), including Bo-EcR-LBD. The alignment includes ecdysone receptors from Lepidoptera [*Heliobius virescens* (F.), *Chilo suppressalis* (Walker), *Bombyx mori* L.], Coleoptera [*Tribolium castaneum* (Herbst), *Tenebrio molitor* L., *Leptinotarsa decemlineata* Say], Hymenoptera [*Apis mellifera* L., *Bombus terrestris* (L.), *Psytalia concolor* (Szépligeti)], Hemiptera [*Nilaparvata lugens* (Stål), *Nezara viridula* (L.), *Bemisia tabaci* (Gennadius)] and Diptera [*Drosophila melanogaster* (Meigen), *Calliphora vicina* (Robineau-Desvoidy), *Aedes aegypti* (L.), *Chironomus tentans* (Fabricius), *Culex pipiens* (L.), *Bradysia coprophila* (Lintner), *Anopheles gambiae* (Giles), *Ceratitis capitata* (Wiedemann), *Bactrocera oleae* (Ross)]. Red dots indicate amino acids that are similar in Dipterans but different from the other insect orders. Green dots indicate amino acids that are similar in Lepidoptera and Diptera but different from the other insect orders. Blue dots indicate amino acids involved in the ligand binding in the EcR-LBD. Amino acid colours indicate similar structure. The figure has been constructed using CINEMA.

ingested via drinking water, 31.8% of adults were killed at 7 days after treatment, and the percentage increased to 98.2% on day 15.

### 3.2 Sequencing of BoEcR-LBD and phylogenetic analysis

The cDNA encoding the BoEcR-LBD was cloned in order to obtain its sequence. However, it was not possible to amplify the 3' end of the transcript. RACE-PCR using the 3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen) did not work. Thus, a consensus sequence of other dipteran species, such as the medfly *Ceratitis capitata* (Wiedemann), *Anopheles gambiae* Giles, *Aedes aegypti* (L.) and *A. albopictus* (Skuse), was used to complete the last six amino acids of helix 12. A multiple alignment with the amino acid sequences of BoEcR-LBD together with the EcR-LBD

from other dipteran species and insects from other orders is shown in Fig. 1. Sequence analysis showed that the EcR-LBD of *B. oleae* exhibits a strong sequence identity with the other members of the Diptera order. In the figure, red dots indicate amino acids that are similar towards dipterans but different from the other insect orders (residues at positions 5, 6 and 11 in helix 1; positions 75, 80 and 117; positions 171 and 179 in helix 9; 236 in helix 12). Other residues are similar in Lepidoptera and Diptera but differ from the other orders. These residues are marked in the figure with green dots (position 19 in helix 1; 71 and 77; 81 and 86 in helix 4; 126; 130 in helix 7; 167 and 169; 173, 180, 184 and 190 in helix 9; 201; 239 in helix 12). In addition, amino acids involved in ligand binding are marked in the figure by blue dots.<sup>14</sup>

### 3.3 Modelling of BoEcR-LBD and docking studies

The BoEcR-LBD, as modelled from the X-ray coordinates of different insect EcR-LBDs, exhibited the canonical three-dimensional conformation of the LBD of arthropod EcR (Fig. 2A). A very similar model was obtained for the fruit fly *Drosophila melanogaster* Meigen DmEcR-LBD (Fig. 2B).<sup>30</sup> Both models readily resemble the tobacco budworm *H. virescens* HvEcR-LBD three-dimensional structure (PDB code 3IXP) used as a template, even though  $\alpha$ -helix H2 was not correctly X-ray solved and is absent from the three-dimensional structure of the HvEcR-LBD (Fig. 2C). Helices H2, H3, H5, H8 and H11 in BoEcR-LBD delineate a ligand-binding cavity that usually accommodates the natural insect ecdysteroid 20E (Fig. 2D) and also the PoA molecule (Fig. 2F). Docking experiments performed with these two ecdysteroids yielded a typical H-bonding scheme that the BoEcR-LBD shares with other arthropod EcR-LBD. Both ecdysteroids interacted with the LBD pocket via a network of six hydrogen bonds involving the hydrophilic residues Glu16, Thr48, Thr51, Ala103 and Tyr113 (Figs 2E and 2G) (Glu21, Thr59, Trh62, Ala114 and Tyr124 in Fig. 1). In addition, stacking interactions occurring with various aromatic residues located at the periphery of the ligand-binding cavity complete the ligand anchorage into the pocket. However, owing to the restricted extent of the ligand-binding cavity, a steric clash occurred with the methoxy-phenyl ring of tebufenozide (Fig. 2H) and methoxyfenozide (Fig. 2I) upon docking of these two agonists to the BoEcR-LBD. In addition, another weak steric hindrance occurred with one of the two methyl groups of the dimethyl-benzoyl ring of both agonists. Although much less severe, a very light steric hindrance still occurred upon docking of the closely related RH-5849 to the BoEcR-LBD (Fig. 2J). These docking experiments suggest that DBH-based insecticides such as tebufenozide and methoxyfenozide readily differ from the agonist RH-5849 in their effects on the insect pest of *B. oleae*, which is in agreement with the previous reported experimental data showing a rather different biological effect of the insecticides in this insect.

## 4 DISCUSSION

In the present study, tebufenozide and methoxyfenozide did not show any deleterious effect on the mortality or the lifespan of *B. oleae* adults, while RH-5849 clearly killed (nearly) all treated olive flies (98–100%), and in the few surviving adults (0–2%) the lifespan was reduced. Deleterious effects of RH-5849 were higher when adults were exposed to treated surfaces than when they ingested the product. The last result was not expected because, although MACs have some topical activity, they primarily act by ingestion.<sup>31</sup>

Studies on larval stages of dipteran species show that tebufenozide, methoxyfenozide and RH-5849 were effective against larvae of the mosquitoes *A. aegypti*, *Culex quinquefasciatus* (Say), *Culex pipiens* L. and *A. gambiae* and of the midges *Chironomus tentans* F.<sup>6–8</sup> In contrast, a lack of activity of tebufenozide on *A. aegypti* larvae was found, while another IGR, pyriproxyfen, proved to be effective against them.<sup>32</sup> Studies carried out with tephritid fruit fly larvae have shown that the IGR lufenuron has negative effects on different parameters when larvae of *C. capitata* and *Bactrocera dorsalis* (Hendel) are fed with the product, but no effects were reported for *Bactrocera cucurbitae* (Coquillett).<sup>33</sup>

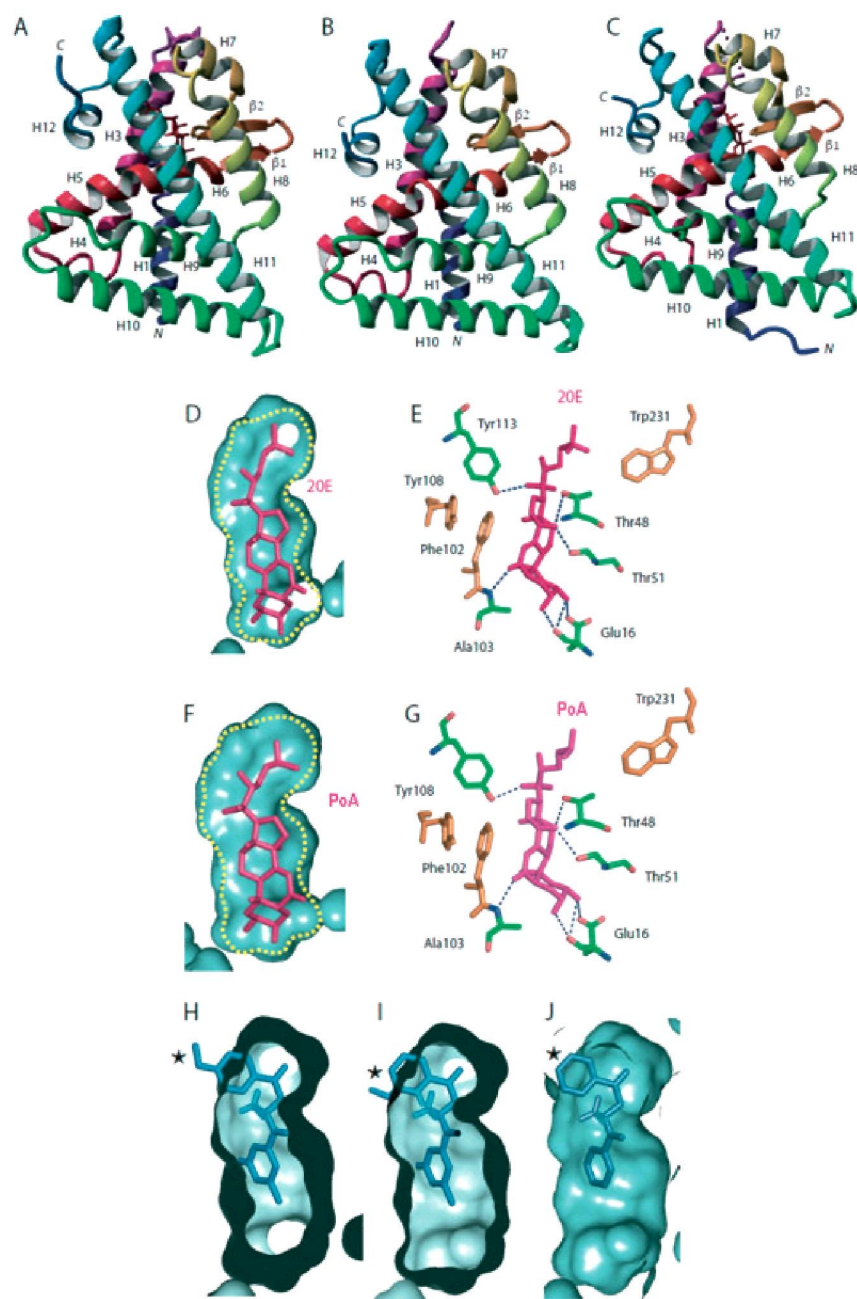
Deleterious effects of IGRs on adults have also been demonstrated on different Tephritidae species. After the ingestion of neem leaf dust and a commercial formulation of neem, a reduction in lifespan of *B. cucurbitae* and *B. dorsalis* was reported.<sup>34</sup>

A high mortality percentage (>75%) after 12–14 days when females of *Anastrepha suspensa* (Loew) ingested diet treated with RH-5849 was also observed,<sup>35</sup> which is in agreement with the present results. In contrast, no effects were observed when *B. oleae* were fed with artificial diet treated with azadirachtin, cyromazine, flufenoxuron and pyriproxyfen, although a slight negative effect on lifespan of adults was reported for lufenuron.<sup>5</sup> The last compound also caused a significant mortality on adults of *Bactrocera latifrons* (Hendel), but had no effects on *C. capitata*, *B. dorsalis* or *B. cucurbitae*.<sup>33</sup>

Different studies have also shown deleterious effects by IGRs on reproductive parameters of Dipterans. Ecdysone has a regulatory role in yolk protein synthesis, and ecdysone agonists would act on this parameter, suppressing egg development.<sup>35</sup> Lawrence<sup>35</sup> reported a suppression of the level of *A. suspensa* egg development and maturation and a reduction in ovary size when RH-5849 was topically applied on females. When RH-5849 was ingested, females were able to oviposit, but on days 8 to 10, 60–75% of the eggs were not viable. It has been observed that neem decreases the fecundity of *B. cucurbitae* and *B. dorsalis* through the block of ovarian development. Similarly, diets treated with neem caused a reduction in fertility of *B. cucurbitae* and *B. dorsalis*.<sup>34,36</sup> Complete egg mortality was also observed when *B. oleae* and *C. capitata* females were fed with diet treated with lufenuron.<sup>5,37</sup> The product also affected the fertility of *B. dorsalis*, *B. latifrons*,<sup>33</sup> *Anastrepha ludens* (Loew.), *Anastrepha obliqua* Mcquart, *Anastrepha serpentina* Wiedemann and *Anastrepha striata* Schiner.<sup>38</sup> However, it had no effects on *B. cucurbitae* fertility or on *C. capitata* fecundity.<sup>33</sup> A lower activity on *B. oleae* and *C. capitata* reproductive parameters was observed for the IGRs cyromazine, azadirachtin, flufenoxuron, triflumuron, diflubenzuron, methoprene, fenoxycarb, buprofezin, benzylphenol J2644 and pyriproxyfen, although pyriproxyfen had no effects on *B. oleae*.<sup>5,37</sup>

In spite of the fact that the EcR-LBD sequence conservation is high among insects, small amino acid substitutions in the sequence can change the three-dimensional structure of the protein, particularly affecting the size and shape of the ligand-binding pocket.<sup>14</sup> The differences in susceptibility for DBH-based ecdysone agonists within insect orders might be due to differences in the structure of the EcR and the binding affinity for the ecdysone agonist ligand molecules.<sup>39–41</sup> BoEcR-LBD exhibits a high conservation among the ligand-binding-involved residues. In Lepidoptera, which show a high sensitivity for tebufenozide and methoxyfenozide, the residues methionine58 and the valines100 and 111 are the divergent residues lining the binding pocket. In BoEcR-LBD these residues are replaced by isoleucine, methionine and isoleucine respectively. These substitutions are also observed in other insect and non-insect arthropods that show no or low susceptibility for tebufenozide and methoxyfenozide,<sup>42</sup> which supports the results obtained in the present biological experiments with these compounds. Wurtz *et al.*<sup>42</sup> also reported that isoleucine58, in particular, generates steric clashes between the  $\gamma$ -methyl group of the isoleucine and the C5-methyl group at the A-ring or the C4-ethyl group at the B-ring of the tebufenozide molecule, depending on the orientation of the insecticide molecule. A steric clash also occurred with the methoxy-phenyl ring of methoxyfenozide. However, in the case of RH-5849, which contains no substitutions on the two benzoyl rings, the steric hindrance occurring upon docking of the products is much less severe (only light) by comparison with the other two DBH-based products. Thus, in this case, the differences in insect susceptibility might be due to the size and shape of the insecticide molecule.





**Figure 2.** Modelling of the olive fly *Bactrocera oleae* EcR-LBD. Overall three-dimensional conformation of the modelled LBD domain of the EcR receptors from the Dipterans *B. oleae* (A) and *Drosophila melanogaster* (B), compared with the LBD structure of the EcR-USP of the lepidopteran *Heliothis virescens* (C). The 12  $\alpha$ -helices distributed along the polypeptide chain are numbered H1 to H12, and the two  $\beta$ -strands,  $\beta$ 1 and  $\beta$ 2, forming a protruding hairpin motif are indicated. N and C consist of the N-terminal and C-terminal ends of the polypeptide chain respectively. (D) Clip view (dashed yellow line) of the ligand-binding pocket of the BoEcR-LBD harbouring 20-hydroxyecdysone (20E) (pink stick). (E) Network of hydrogen bonds (dashed dark lines) anchoring 20E to the BoEcR-LBD. Aromatic residues interacting with the ligand by stacking interactions are coloured orange. Residues are labelled according to the three-dimensional model built for the BoEcR-LBD. (F) Clip view (dashed yellow line) of the ligand-binding pocket of the BoEcR-LBD, harbouring ponasterone A (PA1) (pink stick). (G) Network of hydrogen bonds (dashed dark lines) anchoring PoA to the LBD. Aromatic residues interacting with the ligand by hydrophobic interactions are coloured orange. Residues are labelled according to the three-dimensional model built for BoEcR-LBD. (H) Clip view of the ligand-binding pocket of the BoEcR-LBD harbouring tebufenozide (blue stick). Note the steric clash (★) of the ethyl phenyl ring of tebufenozide with the wall of the ligand-binding pocket. (I) Clip view of the ligand-binding pocket of the BoEcR-LBD harbouring methoxyfenozide (blue stick). Note the steric clash (★) of the methyl-methoxy phenyl ring of methoxyfenozide with the wall of the ligand-binding pocket. (J) Clip view (dashed yellow line) of the ligand-binding pocket of the BoEcR-LBD harbouring the RH-5849 agonist (blue stick). Note the only light steric hindrance (★) of the unsubstituted phenyl ring of RH-5849 with the wall of the ligand-binding pocket.

## 5 CONCLUSIONS

In conclusion, this paper reports on the effect of three IGRs on the olive fruit fly *B. oleae*. The data show no biological activity of methoxyfenozide and tebufenozide, while strong insecticidal effects were found for RH-5849. Modelling and docking experiments also suggest that tebufenozide and methoxyfenozide are not effective against the pest. In contrast, RH-5849 demonstrated a promising activity against *B. oleae*; however, not optimal yet, and more data are needed in other developmental stages to confirm its use. Nonetheless, the authors believe that the present results already imply that the search for and the development of ecdysone agonists to control this pest could be based on the basic lead structure of the molecule of this insecticide. Therefore, it is hoped that such new selective IGRs have a potential in controlling *B. oleae* and could be incorporated into integrated production systems also to counteract the current levels of resistance to products such as dimethoate and spinosad.

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